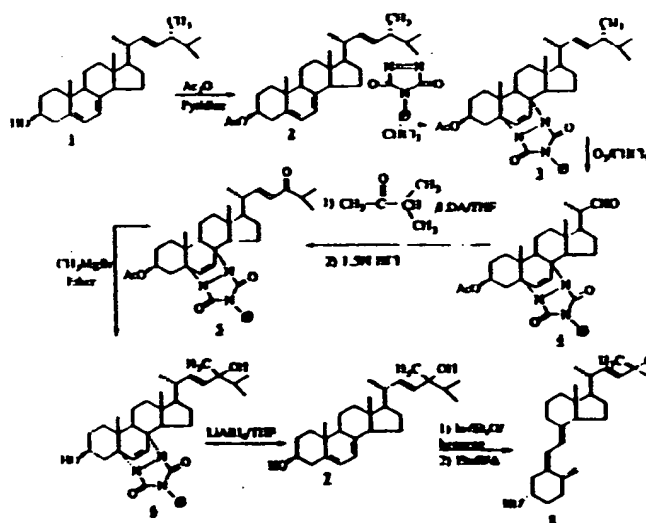




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07J 75/00, A61K 31/59		A1	(11) International Publication Number: WO 92/12165
			(43) International Publication Date: 23 July 1992 (23.07.92)
(21) International Application Number: PCT/US92/00313		(74) Agents: GULBRANDSEN, Carl, E. et al.; 25 West Main Street, Suite 300, P.O. Box 2236, Madison, WI 53701-2236 (US).	
(22) International Filing Date: 7 January 1992 (07.01.92)			
(30) Priority data: 637,867 8 January 1991 (08.01.91) US		(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LU (European patent), MC (European patent), NL (European patent), NO, PL, RU, SE (European patent), US.	
(60) Parent Application or Grant (63) Related by Continuation US 637,867 (CIP) Filed on 8 January 1991 (08.01.91)			
(71) Applicant (for all designated States except US): LUNAR CORPORATION [US/US]; 313 West Behtline Highway, Madison, WI 53713 (US).		Published With international search report.	
(72) Inventors; and (75) Inventors/Applicants (for US only): BISHOP, Charles, W. [US/US]; 3641 Okanogan Court, Verona, WI 53593 (US). JONES, Glenville [CA/CA]; 112 Westmorland Road, Kingston, Ontario (CA). KNUTSON, Joyce, C. [US/US]; 24 North Prospect, Madison, WI 53705 (US). MORIARTY, Robert, M. [US/US]; 1030 Erie Street, Oak Park, IL 60302 (US). PENMASTA, Raju [IN/US]; 493 West St. Charles, Elmhurst, IL 60126 (US). STRUGNELL, Stephen [CA/CA]; 15 MacPherson Avenue #13-303, Kingston, Ontario (CA).			

(54) Title: METHODS FOR PREPARATION AND USE OF 1 α ,24-DIHYDROXY VITAMIN D₂

(57) Abstract

1 α ,24-dihydroxy vitamin D₂ which is useful as an active compound of pharmaceutical compositions for the treatment of disorders of calcium metabolism and for various skin disorders. The invention also includes preparation of synthetic 1 α ,24-dihydroxy vitamin D₂ starting from ergosterol which is converted in six steps to 24-hydroxyergosterol. 24-hydroxyergosterol is irradiated and thermally converted to 24-hydroxy vitamin D₂ which is converted in five steps to 1 α ,24-dihydroxy vitamin D₂. The syntheses also produce novel intermediates.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

TITLE: Methods for preparation and use of 1 α ,24 dihydroxy Vitamin D₂

TECHNICAL FIELD

This invention relates to biologically active vitamin D₂ compounds. More specifically, this invention relates to the hormonally active, natural metabolite 1 α ,24 dihydroxy vitamin D₂ and to methods of preparing this metabolite. This invention also relates to a pharmaceutical composition which includes a pharmaceutically effective amount of 1 α ,24-dihydroxy vitamin D₂, and to a method of controlling abnormal calcium metabolism by administering a pharmaceutically effective amount of the compound.

BACKGROUND OF THE INVENTION

Vitamin D and its active metabolites are known to be important in regulating calcium metabolism in animals and humans. The naturally occurring form of vitamin D in animals and humans is vitamin D₃. It has been shown that in animals, including humans, vitamin D₃ is activated by being hydroxylated in the C₂₅ position in the liver, followed by 1 α -hydroxylation in the kidney to produce the hormone 1 α ,25-dihydroxy vitamin D₃ ["1,25-(OH)₂D₃"]. See, U.S. Patent No. 3,880,894. The major physiological pathway for catabolism of the vitamin D₃ metabolites, 25-hydroxy vitamin D₃ and 1,25-(OH)₂D₃ is initiated by C₂₄-oxidation. Holick, M.F., Kleiner-Bossallier, A., Schnoes, H.K., Kasten, P.M., Boyle, I.T., and DeLuca, H.F., J. Biol. Chem., 248, 6691-6696 (1973).

Vitamin D₂ is the major, naturally occurring form of vitamin D found in plants. Vitamin D₂ differs structurally from vitamin D₃ in that vitamin D₂ has a methyl group at C₂₄ and has a double bond between C₂₂ and C₂₃.

Shortly after their discovery, it seemed apparent that vitamin D₂ and vitamin D₃ had similar, if not equivalent, biological activity. It has also been commonly believed that the metabolism (i.e., the activation and catabolism) of vitamin D₂ was the same as for vitamin D₃. See, Harrison's Principles of

-2-

Internal Medicine: Part Seven, "Disorders of Bone and Mineral Metabolism: Chap. 35," in E. Braunwald, K.J. Isselbacher, R.G. Petersdorf, J.D. Wilson, J.B. Martin and H.S. Fauci (eds.), Calcium, Phosphorus and Bone Metabolism: Calcium Regulating Hormones, McGraw-Hill, New York, pp. 1860-1865. In this regard, the active form of vitamin D₂ is believed to be 1 α ,25-dihydroxy vitamin D₂ ["1,25-(OH)₂D₂"]. Further, 24-hydroxy derivatives of 25-hydroxy vitamin D₂ and 1,25-(OH)₂D₂, that is, 24,25-dihydroxy vitamin D₂ and 1 α ,24,25-trihydroxy vitamin D₂, are known, suggesting that catabolism of vitamin D₂, like vitamin D₃, proceeds through the same C₂₄ oxidation step. Jones, G., Rosenthal, D., Segev, D., Mazur, Y., Frolow, F., Halfon, Y., Robinavich, D. and Shakked, Z., Biochemistry, 18:1094-1101 (1979).

It has recently been found, however, that an active analogue of vitamin D₂, 1 α -hydroxy vitamin D₂ ["1 α -(OH)D₂"] has pharmacological properties distinctly different than those exhibited by its vitamin D₃ counterpart, 1 α -hydroxy vitamin D₃ ["1 α -(OH)D₃"]. Commonly assigned U.S. Patent Application 07/569,412 discloses that 1 α -(OH)D₂ will reverse the loss of bone mass in human osteoporotic patients when administered at dosages of 2.0 μ g/day or higher. Because of toxicity, dosage levels of 2.0 μ g/day or greater are not safely obtained with 1 α -(OH)D₃.

Such distinct pharmacological properties may be explained by the present inventors' discovery that pharmacological dosages of 1 α -(OH)D₂ administered to humans are metabolized in part to biologically active 1 α ,24-dihydroxy vitamin D₂ ["1 α ,24-(OH)₂D₂"]. As explained in more detail below, the hydroxylation at the 24 carbon position of the 1-hydroxylated vitamin D₂ molecule, represents an activation pathway peculiar to the vitamin D₂ molecule.

While 1 α ,24-dihydroxy vitamin D₂ ["1 α ,24-(OH)₂D₂"] has been chemically synthesized (U.S. Patent No. 4,022,891) it has not been demonstrated to be a natural compound in biological systems. Furthermore, the present inventors have discovered that 1 α ,24-(OH)₂D₂ has distinctly different biological activity from that exhibited by 1 α ,24-(OH)D₂. For example, Ishizuka et al., have found that 1 α ,24-(OH)₂D₂ binds the 1,25-(OH)₂D₃ receptor site more tightly than does 1,25-(OH)₂D₃ itself. Ishizuka, S.,

-3-

Bannai, K., Naruchi, T. and Hashimoto, Y., Steroids, 37:1,33-42 (1981); Ishizuka, S., Bannai, K., Naruchi, T. and Hashimoto, Y., Steroids, 39:1,53-62 (1982). Using a similar assay, the present inventors have discovered that the $1\alpha,24-(OH)_2D_3$ is two-fold less competitive in binding the $1,25-(OH)_2D_3$ receptor site than is $1,25-(OH)_2D_3$. The present inventors have also found that $1\alpha,24-(OH)_2D_3$ shows a relatively poor binding affinity for the vitamin D serum binding protein of blood which is evidence of a rather short half life indicative of low toxicity.

The present inventors have demonstrated the presence of circulating $1\alpha,24-(OH)_2D_3$ in humans administered $1\alpha-(OH)D_3$. This indicates that in animals and man, vitamin D_3 is naturally metabolized to both $1,25-(OH)_2D_3$ and $1\alpha,24-(OH)_2D_3$. The relative ratios of the two vitamin D_3 hormones appear to vary according to the precursor and the amount of precursor presented to the C_{25} pathway. Thus it appears that as dosages of $1\alpha-(OH)D_3$ are increased, the ratio of $1\alpha,24-(OH)_2D_3$ to $1,25-(OH)_2D_3$ increases.

These results which are presented in more detail below, indicate that $1\alpha,24-(OH)_2D_3$ has the desirable characteristic of high biological activity with low toxicity. The fact that $1\alpha,24-(OH)_2D_3$ is a significant metabolite when pharmacological levels of $1\alpha-(OH)D_3$ are administered indicates that $1\alpha,24-(OH)_2D_3$ may be mediating the desirable pharmacological effects of $1\alpha-(OH)D_3$ and is a useful therapeutic drug for treating various types of disorders involving calcium metabolism.

SUMMARY OF THE INVENTION

The invention provides synthetic $1\alpha,24-(OH)_2D_3$ which is a bioactive form of vitamin D_3 . The hormone may also be referred to as $1\alpha,24$ -dihydroxy ergocalciferol and is represented by the structure given hereinafter. The compound has potent biological activity and rapid systemic clearance, indicating low toxicity.

The invention also encompasses a novel method of producing $1\alpha,24$ dihydroxy vitamin D_3 which entails using ergosterol as a starting material, forming the 24-hydroxy vitamin D_3 and then, 1α -hydroxylating the 24-hydroxy compound. In the course of this synthesis, novel intermediates are also produced.

The compound of the invention is useful in the treatment of various diseases characterized by vitamin D deficiency and

-4-

various bone depletive disorders, in particular, treatment without the concomitant incidence of hypercalcemia or hypercaluria. The compounds of the invention are advantageously used as active compounds of pharmaceutical compositions for vitamin D deficiency diseases, for reversing or preventing the loss of bone mass or bone mineral content in persons predisposed to developing such loss, and for stabilizing bone density in persons suffering from renal osteodystrophy.

The compound of the invention is also useful as topical agents for treatment of certain skin disorders. The compound of the invention as advantageously used as an active ingredient for topical compositions which may also include other agents capable of ameliorating skin disorders.

Other advantages and a fuller appreciation of the specific adaptations, compositional variations, and physical and chemical attributes of the present invention will be gained upon an examination of the following detailed description of the invention, taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will hereinafter be described in conjunction with the appended drawings, wherein like designations refer to like elements throughout and in which:

Figure 1 illustrates preparative steps for the synthesis of 24-hydroxy vitamin D₂; and

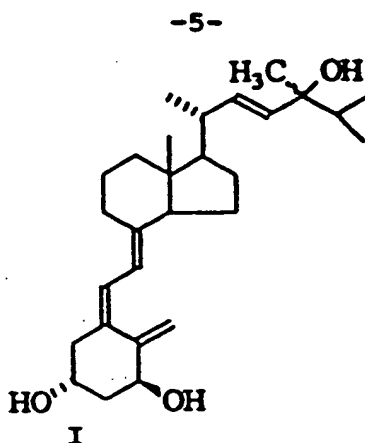
Figure 2 illustrates preparative steps for the synthesis of 1 α ,24 dihydroxy vitamin D₂ starting with 24-hydroxy vitamin D₂.

DETAILED DESCRIPTION

The present invention provides synthetic 1 α ,24-dihydroxy vitamin D₂ [1 α ,24-(OH)₂-D₂].

As used herein, the terms "biological activity", "biologically active", "bioactive", or "biopotent" are meant to refer to biochemical properties of compounds such as affecting metabolism, e.g., affecting serum calcium concentration, or binding to an appropriate receptor protein, e.g., binding to vitamin D receptor protein.

In one of its aspects, the invention encompasses the biologically active compound of the general formula (I):



In another aspect, the invention involves the preparation of compound of formula (I). Synthesis of 1 α ,24-dihydroxy vitamin D₂ is accomplished according to the schema presented in Figures 1 and 2. As seen in Figure 1, the synthesis uses ergosterol as the starting material. Ergosterol is converted to 24-hydroxyergosterol (5,7,22 ergostatriene-3 β ,24-diol (7)) by a six-step process. The 24-hydroxy ergosterol is then irradiated and thermally converted by methods well known in the art to yield 24-hydroxy vitamin D₂. As seen in Figure 2, 24-hydroxy vitamin D₂ is then hydroxylated in a six-step process to yield 1 α ,24 dihydroxy vitamin D₂, using a procedure similar to that described by Paaren, et al., *J. Org. Chem.*, vol. 45, p. 3253 (1980).

Specifically, ergosterol is acetylated to form the 3β -acetate (2). An adduct (3) is then formed with the B-ring of the ergosterol structure by reaction of the 3β -acetate with a triazoline dione. The adduct (3) is then ozonated to truncate the side chain to form a C-21 aldehyde (4). The side chain is reestablished by reaction of the resulting aldehyde with the appropriate keto-compound to yield the 24-enone (5). The enone is then converted to the 24-methyl, 3β ,24-dihydroxy adduct (6). This adduct is then reacted with a lithium aluminum hydride to deprotect the adduct and yield 24-hydroxy ergosterol (7). The 24-hydroxy ergosterol is then irradiated and thermally treated to form 24-hydroxy vitamin D_2 . The 24-hydroxy vitamin D_2 is then tosylated to yield 3β -tosylate of the 24-hydroxy vitamin D_2 . The tosylate is displaced by solvolysis to yield the 6-methoxy-24-hydroxy-3,5-cyclo vitamin D_2 . The cyclovitamin D_2 is subjected to allylic oxidation to form the 1α , 24-dihydroxy cyclovitamin derivative. The 1α ,24 dihydroxy cyclovitamin derivative is sequentially solvolized and subjected to a Diels-Alder type

-6-

reaction which removes the 6-methoxy group and separates the 1 α ,24 dihydroxy vitamin D₂ (5,6 cis) from the 5,6 trans 1 α ,24 dihydroxy vitamin D₂.

The compound of the invention is applicable to various clinical and veterinary fields, and is particularly useful for the treatment of abnormal metabolism of calcium and phosphorus. Specifically, the compound of formula (I) is intended to be used, for example, to stimulate osteoblastic activity, as measured by serum levels of osteocalcin. Osteocalcin is one of the major proteins in the bone matrix. The compound of formula (I) binds to the vitamin D serum binding protein more weakly than does 1,25-(OH)₂ D₃, indicative of rapid clearance and low toxicity, which enhances its pharmaceutical properties.

In a further aspect, the invention entails a method of controlling calcium metabolism, such as for treating abnormal calcium metabolism caused, e.g., by liver failure, renal failure, gastrointestinal failure, etc. The compounds of formula (I) can be used to treat prophylactically or therapeutically vitamin D deficiency diseases and related diseases, for example, renal osteodystrophy, steatorrhea, anticonvulsant osteomalacia, hypophosphatemic vitamin D-resistant rickets, osteoporosis, including postmenopausal osteoporosis, senile osteoporosis, steroid-induced osteoporosis, and other disease states characteristic of loss of bone mass, pseudodeficiency (vitamin D-dependent) rickets, nutritional and malabsorptive rickets, osteomalacia and osteopenias secondary to hypoparathyroidism, post-surgical hypoparathyroidism, idiopathic hypothyroidism, pseudoparathyroidism, and alcoholism.

1 α ,24 dihydroxy vitamin D₂ is also of value for the treatment of hyperproliferative skin disorders such as psoriasis, eczema, lack of adequate skin firmness, dermal hydration, and sebum secretion.

The compound of formula (I) is useful as an active compound in pharmaceutical compositions having reduced side effects and low toxicity as compared with the known analogs of active forms of vitamin D₂, when applied, for example, to diseases induced by abnormal metabolism of calcium. These pharmaceutical compositions constitute another aspect of the invention.

The pharmacologically active compound of this invention can be processed in accordance with conventional methods of pharmacy to produce medicinal agents for administration to patients, e.g., mammals including humans. For example, the compound of formula (I) can be employed in admixtures with conventional excipients, e.g., pharmaceutically acceptable carrier substances suitable for enteral (e.g., oral), parenteral, or topical application which do not deleteriously react with the active compound.

Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohols, gum arabic, vegetable oils (e.g., almond oil, corn oil, cottonseed oil, peanut oil, olive oil, coconut oil), mineral oil, fish liver oils, oily esters such as Polysorbate 80, polyethylene glycols, gelatine, carbohydrates (e.g., lactose, amylose or starch), magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, etc.

The pharmaceutical preparations can be sterilized and, if desired, be mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or one or more other active compounds, for example, vitamin D₃ and its 1 α -hydroxylated metabolites, conjugated estrogens or their equivalents, anti-estrogens, calcitonin, biphosphonates, calcium supplements, cobalamin, pertussis toxin and boron.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solution, as well as suspensions, emulsions, or implants, including suppositories. Parenteral administration suitably includes subcutaneous, intramuscular, or intravenous injection, nasopharyngeal or mucosal absorption, or transdermal absorption. Ampoules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees, liquids, drops, suppositories, lozenges, powders, or capsules. A syrup, elixir, or the like can be used if a sweetened vehicle is desired.

For topical application, suitable nonsprayable viscous, semi-solid or solid forms can be employed which include a carrier

-8-

compatible with topical application and having a dynamic viscosity preferably greater than water, for example, mineral oil, almond oil, self-emulsifying beeswax, vegetable oil, white soft paraffin, and propylene glycol. Suitable formulations include, but are not limited to, creams, ointments, lotions, solutions, suspensions, emulsions, powders, liniments, salves, aerosols, transdermal patches, etc., which are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, demulsifiers, wetting agents, etc. A cream preparation in accordance with the present invention suitably includes, for example, mixture of water, almond oil, mineral oil and self-emulsifying beeswax; an ointment preparation suitably includes, for example, almond oil and white soft paraffin; and a lotion preparation suitably includes, for example, dry propylene glycol.

Topical preparations of the compound in accordance with the present invention useful for the treatment of skin disorders may also include epithelialization-inducing agents such as retinoids (e.g., vitamin A), chromanols such as vitamin E, β -agonists such as isoproterenol or cyclic adenosine monophosphate (cAMP), anti-inflammatory agents such as corticosteroids (e.g., hydrocortisone or its acetate, or dexamethasone) and keratoplastic agents such as coal tar or anthralin. Effective amounts of such agents are, for example, vitamin A about 0.003 to about 0.3% by weight of the composition; vitamin E about 0.1 to about 10%; isoproterenol about 0.1 to about 2%; cAMP about 0.1 to about 1%; hydrocortisone about 0.25 to about 5%; coal tar about 0.1 to about 20%; and anthralin about 0.05 to about 2%.

For rectal administration, the compound is formed into a pharmaceutical composition containing a suppository base such as cacao oil or other triglycerides. To prolong storage life, the composition advantageously includes an antioxidant such ascorbic acid, butylated hydroxyanisole or hydroquinone.

For treatment of calcium metabolic disorders, oral administration of the pharmaceutical compositions of the present invention is preferred. Generally, the compound of this invention is dispensed by unit dosage form comprising about 0.5 μ g to about 25 μ g in a pharmaceutically acceptable carrier per unit dosage. The dosage of the compound according to this

-9-

invention generally is about 0.01 to about 1.0 $\mu\text{g/kg/day}$, preferably about 0.04 to about 0.3 $\mu\text{g/kg/day}$.

For topical treatment of skin disorders, the dosage of the compound of the present invention in a topical composition generally is about 0.01 μg to about 10 μg per gram of composition.

It will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the efficacy of the specific compound employed, the particular compositions formulated, the mode of application, and the particular situs and organism being treated. For example, the specific dose for a particular patient depends on the age, body weight, general state of health and sex, on the diet, on the timing and mode of administration, on the rate of excretion, and on medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given host can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compounds and of a known agent, such as by means of an appropriate conventional pharmacological protocol.

In a still further aspect, the compound of the present invention can also be advantageously used in veterinary compositions, for example, feed compositions for domestic animals to treat or prevent hypocalcemia. Generally, the compound of the present invention is dispensed in animal feed such that normal consumption of such feed provides the animal about 0.01 to about 1.0 $\mu\text{g/kg/day}$.

The following examples are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. In the following examples proton nuclear magnetic (^1H NMR) spectra were recorded with a Bruker AM--400(400 MHz) with aspect 3000 Computer in CDCl_3 , solutions with CHCl_3 , as an internal standard. Chemical shifts are reported in ppm. Ultraviolet spectra were recorded with a Hitachi U-2000 Spectrophotometer and are reported for ethanol solutions.

-10-

Example 1: Generation, purification and identification of $1\alpha,24-(OH)_2D_2$ in human liver cells incubated with $1\alpha-(OH)D_2$

Substantially pure $1\alpha-(OH)D_2$ was obtained from Bone Care International, Inc. of Madison, Wisconsin. The $1\alpha-(OH)D_2$ was cultured with Hep 3B cells, derived from a human hepatoma using known methods in the art.

Lipid extracts were generated by known methods in the art and were subjected to high pressure liquid chromatography (HPLC) on Zorbax-SiL developed with hexane/isopropanol/methanol (91:7:2). The putative $1\alpha,24-(OH)_2D_2$ metabolite eluted between the parent $1\alpha-(OH)D_2$ and standard $1,25-(OH)_2D_2$ (also obtained from Bone Care International, Inc. of Madison, Wisconsin). The $1\alpha,24-(OH)_2D_2$ was further purified by this HPLC system before the metabolite's identification was undertaken using mass spectrometry analysis.

The purified metabolite was more polar than the starting material, $1\alpha-(OH)D_2$ and thus was tentatively concluded to be a dihydroxy vitamin D_2 metabolite. This metabolite also possessed the vitamin D chromophore, indicating retention of the cis-triene system of vitamin D. Since the metabolite was derived from $1\alpha-(OH)D_2$, its structure was thus $1\alpha,X-(OH)_2D_2$.

The trimethylsilyl-derivative of the $1\alpha,X-(OH)_2D_2$ was prepared according to known methods in the art and mass spectrometry was performed on the TMS-derivative and the native compound. The TMS-derivative was analyzed by GC-MS, and the identification was mainly derived from interpretation of the fragmentation pattern of the pyro-metabolite. The molecular ion possessed a m/z of 644 indicating a dihydroxyvitamin D_2 with addition of three TMS groups accounting for 216 units of additional mass. Since $1\alpha-(OH)D_2$ has 3β - and 1α - groups and the putative metabolite had one additional hydroxyl, all three hydroxyls were thus derivatized. Distinctive fragments were found at m/z 601, 511, 421, 331 representing loss of a 43 mass unit of fragment alone or in addition to one, two or three TMS groups of 90 units each. This pattern was most likely explained by cleavage of the C-24 to C-25 bond loss of C_5H_7 accounting for 43 mass units. This represents loss of the $C_{24}-C_{25}-C_{27}$ fragment.

-11-

Furthermore, the mass spectrum lacked the m/z 131 fragment characteristic of all 25-hydroxylated vitamin D compounds.

The mass spectrum showed the m/z 513 fragment indicating loss of 131 mass units due to A-ring cleavage with loss of $C_1-C_3-C_4$, also characteristic of vitamin D compounds. The mass spectrum also contained m/z 143 which was probably derived from C-24 to C-23 cleavage and a loss of a methyl group. The unusual loss of 43 units indicating $C_{24}-C_{25}$ fragility coupled with the loss of a fragment due to $C_{23}-C_{24}$ cleavage indicated that the extra hydroxyl in $1\alpha, X-(OH)_2D_2$ was at carbon-24. Thus, the structure was identified as $1\alpha, 24-(OH)_2D_2$.

The native metabolite was analyzed by direct probe mass spectrometry. This analysis was consistent with a hydroxyl in the 24 position, and was also consistent with the GC-MS analysis of the TMS-derivative described above. The native metabolite showed the expected molecular ion at m/z 428 and a distinctive fragment at m/z 367, indicating the loss of one water and the $C_{23}-C_{24}-C_{25}$ fragment of 43 mass units.

Example 2: Synthesis of $1\alpha, 24$ dihydroxy vitamin D_2

(22E)-5,7,22-ergostatriene-3 β -yl acetate (2)

To a solution of 50 gm (0.13 mol) of ergosterol (1) in 300 ml of anhydrous pyridine was added 33.3 ml (0.35 mol) of acetic anhydride. The mixture was stirred at room temperature overnight and then 600 ml of water was added. The precipitate was filtered and washed three times with 200 ml portions of acetonitrile and then air dried to yield 42.0 g (74%) of (2).

22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-cholesterol-3 β -yl acetate (4)

To a solution of 33.0 g (0.075 mol) of ergosterol acetate (2) in 1000 ml of chloroform was added 13.2 g (0.075 mol) of 4-phenyl-1,2,4-triazoline-3,5-dione. The solution of the thus formed (3) was stirred at room temperature for 30 min. and then 5 ml of pyridine was added. The solution was cooled to -78°C and treated at -78°C with an ozone-oxygen mixture for 2 hours and then thoroughly purged with nitrogen. Then 50 ml of dimethylsulfide was added and the mixture was washed with 300 ml of water, then twice with 200 ml of 2N HCl and finally 300 ml of

-12-

water. The organic layer was separated, dried over anhydrous MgSO_4 , and concentrated to dryness in vacuo. The residue was purified on a silica gel column using 30% ethyl acetate in hexane to yield 16.0 g (39%) of the title compound as a foamy solid.

^1H NMR: (400 MHz; CDCl_3): δ ppm 0.85 (3H, s, 18- CH_3), 1.10 (3H, s, 19- CH_3), 1.15 (3H, d, 21- CH_3), 1.99 (3H, s, 3 β - CH_3CO), 5.45 (1H, m, 3 α -H), 6.26 (1H, d, 7-H), 6.40 (1H, d, 6-H), 7.42 (5H, m, Ph), 9.58 (1H, d, HCO).

(22E)5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)cholesta-6,22-diene-24-one-3 β -yl acetate (5)

Butyllithium (1.6M solution in hexane 8.94 ml, 0.014 mol) was added to a stirred, cooled (0°C) solution of diisopropylamine (1.45 g, 0.014 mol) in dry tetrahydrofuran (20 ml) under nitrogen. 3-Methylbutan-2-one (1.23 g, 0.014 mol) in dry tetrahydrofuran (6 ml) was added dropwise at 0°C over 15 min. The solution was stirred at 0°C for 1 hr. more, then cooled to -70°C and a solution of the aldehyde (4) (6.0 g, 0.011 mol) in dry tetrahydrofuran (60 ml) was added. The temperature was raised to -20°C and kept at this temperature for 3 hrs. Then glacial acetic acid (20 ml) was added at -20°C and the solution was brought to room temperature. Ether (800 ml) and water (400 ml) were added and the organic layer was separated and washed with 10% hydrochloric acid (2x300 ml), saturated sodium bicarbonate solution (2x300 ml), and water (2x300 ml). Concentration gave the crude product (7.5 g) which was dissolved in tetrahydrofuran (100 ml) containing 1.5 N-hydrochloric acid (12 ml). After refluxing for 1.5 hrs., the mixture was diluted with ether (600 ml), washed with a 5% sodium carbonate solution (2x200 ml) and water (2x200 ml), and dried (anhyd. MgSO_4). Concentration under reduced pressure gave the crude product (7.0 g). Chromatography over silica gel (50% ethyl acetate in hexane) gave the enone (5) 4.0 g (59%).

^1H NMR: (400 MHz): δ ppm 0.83 (3H, s, 18- CH_3), 0.99 (3H, s, 19- CH_3), 1.09 (6H, dd, 26 and 27- CH_3), 1.12 (3H, d, 21- CH_3), 2.0 (3H, s, 3 β - CH_3CO), 2.84 (1H, m, 25-H), 5.45 (1H, m, 3 α -H), 6.06 (1H, d, 23-H), 6.24 (1H, d, 7-H), 6.39 (1H, d, 6-H), 6.71 (1H, dd, 22-H), 7.42 (5H, m, Ph).

-13-

(22E)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol (6)

The enone (5) (3.5 g, 5.7 mmol) in dry ether (100 ml) was cooled to 0°C and methylmagnesium bromide (3.0 M solution in ether 6.8 ml, 0.02 mol) was added dropwise. After 1 hr. at 0°C, saturated ammonium chloride (100 ml) was added. The organic layer was separated. The aqueous layer was extracted with ether (2x200 ml). The combined ether phases were dried over anhydrous MgSO₄ and concentrated to dryness in vacuo to yield the crude product 3.0 g (90%) of (6).

(22E)-5,7,22-ergostatriene-3 β ,24-diol (7)

To a solution of 3.0 g (5.1 mmol) of (6) in dry tetrahydrofuran (250 ml) was added 3.6 g (0.09 mol) of lithium aluminum hydride. The mixture was heated under reflux for 3 hrs., cooled with ice water bath and reaction mixture decomposed by the cautious dropwise addition of ice water (5 ml). The mixture was filtered and the filtrate was concentrated in vacuo to remove most of the tetrahydrofuran. The residue was dissolved in 200 ml of ethyl acetate and washed twice with saturated NaCl solution (2x200 ml), dried over anhyd. MgSO₄ and concentrated in vacuo. The residue was purified on a silica gel column using 30% ethyl acetate in hexane to yield 1.5 g (71%) of (7).

¹H NMR: (400 MHz, CDCl₃): δ ppm 0.64 (3H, s, 18-H), 0.88 (6H, dd, 26 and 27-CH₃), 0.93 (3H, s, 19-CH₃), 1.06 (3H, d, 21-CH₃), 1.19 (3H, s, 28-CH₃), 3.55 (1H, m, 3 α -H), 5.36 (1H, d, 7-H), 5.42 (2H, m, 22 and 23-H), 5.52 (1H, d, 6-H). UV (ethanol) λ_{max} : 282 nm.

24-hydroxyvitamin D₂ (8)

One gram (2.4 mmol) of (7) was dissolved in 250 ml of ether and benzene (4:1) and irradiated with stirring under nitrogen in a water-cooled quartz immersion well using a Hanovia medium-pressure UV lamp for 2 hrs. The solution was concentrated in vacuo, redissolved in 100 ml of ethanol and heated under reflux overnight. The solution was concentrated to dryness in vacuo and the residue was purified on a silica gel column using 30% ethyl acetate in hexane to yield 0.55 g (55%) of (8).

-14-

¹H NMR: (400 MHz, CDCl₃): δppm 0.57 (3H, s, 18-CH₃), 0.92 (6H, dd, 26 and 27-CH₃), 1.06 (3H, d, 21-CH₃), 1.20 (3H, s, 28-CH₃), 3.93 (1H, m, 3-H), 4.79 (1H, m (sharp), 19-H), 5.01 (1H, m, (sharp), 19-H), 5.43 (2H, m, 22 and 23-H), 6.02 (1H, d, 7-H), 6.22 (1H, d, 6-H). UV (ethanol) λ_{max}: 265 nm.

24-hydroxyvitamin D₂ tosylate (9)

To a solution of 0.55 g (1.3 mmol) of (8) dissolved in 5 ml of anhydrous pyridine was added 0.6 g (3.2 mmol) of tosyl chloride. The mixture was stirred under nitrogen at 5°C for 20 hrs. The reaction mixture was poured into 100 ml of cold saturated NaHCO₃ solution and extracted with ether (3x100 ml). The combined organic extracts were washed with 5% HCl solution (2x200 ml) saturated sodium bicarbonate solution (2x200 ml) and saturated NaCl solution (2x200 ml), dried over anhyd. MgSO₄ and concentrated in vacuo to yield 0.62 g (84%) of (9).

¹H NMR: (400 MHz, CDCl₃): δppm 0.57 (3H, s, 18-CH₃), 0.92 (6H, dd, 26 and 27-CH₃), 1.08 (3H, d, 21-CH₃), 1.24 (3H, s, 28-CH₃), 2.43 (3H, s, CH₃ (tosylate)), 4.69 (1H, m, 3-H), 4.77 (1H, m, (sharp), 19-H), 5.0 (1H, m, (sharp), 19-H), 5.42 (2H, m, 22 and 23-H), 6.03 (1-H, d, 7-H), 6.25 (1-H, d, 6-H) 7.31 and 7.83 (4H, d, aromatic).

24-hydroxy-3,5-cyclovitamin D₂ (10)

To a solution of 0.6 g (1.06 mmol) of (9) dissolved in 50 ml of anhydrous methanol was added sodium bicarbonate 4.0 (0.047 mol). The mixture was heated at reflux for 6 hrs. The reaction mixture was concentrated in vacuo. Water (100 ml) was added followed by extraction with ether (2x200 ml). The combined ether extracts were dried over anhydrous MgSO₄ and concentrated to dryness in vacuo to yield 450 mg (100%) of (10) as an oil.

1α,24-dihydroxy-3,5-cyclovitamin D₂ (11)

tert-Butyl hydroperoxide (870 μl (2.61 mmol); 3M in toluene) was added to a suspension of 73 mg (0.66 mmol) of selenium dioxide in 50 ml of anhydrous dichloromethane under nitrogen. The mixture was stirred at room temperature under nitrogen for 3 hrs. Then 0.1 ml of anhydrous pyridine was added followed by a

-15-

solution of 450 mg (1.06 mmol) of (10) dissolved in 15 ml of anhydrous dichloromethane. The mixture was stirred under nitrogen at room temperature for 10 min. then 25 ml of 10% NaOH solution was added and the mixture was extracted with ether (3x100 ml). The combined ether extracts were washed with 10% NaOH solution (2x100 ml), water (2x100 ml), saturated sodium chloride solution (2x100 ml), dried over anhydrous MgSO_4 and concentrated to dryness in vacuo. The residue was purified on a silica gel column using a mixture of 30% ethyl acetate in hexane to yield 110 mg (24%) of (11).

^1H NMR: (400 MHz, CDCl_3): δ ppm, 0.55 (3H, s, 18-CH_3), 0.90 (6H, dd, 26 and 27- CH_3), 1.03 (3H, d, 21- CH_3), 1.19 (3H, s, 28- CH_3), 3.25 (3H, s, $-\text{OCH}_3$), 4.19 (1H, d, 6-H), 4.19 (1H, m, 1-H), 4.92 (2H, d, 7-H), 5.15 (1H, m, (sharp), 19-H), 5.2 (1H, m, (sharp), 19-H), 5.42 (2H, m, 22 and 23-H).

5,6-cis and 5,6-trans-1 α ,24-dihydroxyvitamin D_2 (12, 13)

1 α ,24-dihydroxy-3,5-cyclovitamin D_2 (11) 110 mg (0.25 mmol) was dissolved in 2.0 ml of dimethylsulfoxide and 1.5 ml of acetic acid and heated at 50°C under nitrogen for 1 hr. The solution was poured over ice and 50 ml of saturated NaHCO_3 solution. The mixture was extracted with ether (3x100 ml). The combined ether extracts were washed with saturated NaHCO_3 solution (3x100 ml), water (2x100 ml), saturated NaCl solution (2x200 ml), dried over anhyd. MgSO_4 and concentrated in vacuo to yield the crude product 100 mg (93%) of (12) and (13).

5,6-cis-1 α ,24-dihydroxyvitamin D_2 (12)

To a solution of (12) and (13) in 5 ml of ethyl acetate was added 20 mg (0.2 mmol) of maleic anhydride and the mixture was stirred at 35°C for 24 hrs. under nitrogen. The solution was concentrated to dryness in vacuo. The residue was purified on a silica gel column using 50% ethyl acetate in hexane to yield 20 mg (22%) of (12).

^1H NMR: (400 MHz, CDCl_3): δ ppm 0.57 (3H, s, 18-CH_3), 0.89 (6H, dd, 26 and 27- CH_3), 1.04 (3H, d, 21- CH_3), 1.21 (3H, s, 28- CH_3), 4.23 (1H, m, 3-H), 4.40 (1H, m, 1-H), 5.0 (1H, m, (sharp), 19-H), 5.33 (1H, m, (sharp), 19-H), 5.44 (2H, m, 22 and

-16-

23-H), 6.01 (1H, d, 7-H), 6.37 (1H, d, 6-H). UV (ethanol) λ_{max} : 265 nm.

Example 3: Preferential production of $1\alpha,24\text{-(OH)}_2\text{D}_2$ with increased substrate concentrations in vitro.

Hep 3B cells were incubated with $1\alpha\text{-OH-D}_2$ as described above, at final concentrations of 1, 10, or 100 nM (Experiment 1), and 1 or 10 μM (Experiment 2) and $1\alpha,24\text{-(OH)}_2\text{D}_2$ was extracted and purified. The $1\alpha,24\text{-(OH)}_2\text{D}_2$ and $1,25\text{-(OH)}_2\text{D}_2$ metabolites were quantitated by recovered radiolabel (Experiment 1) or by photodiode array spectrophotometry (Experiment 2). As shown in Table 1, the amount of $1\alpha,24\text{-(OH)}_2\text{D}_2$ increased relative to the amount of $1,25\text{-(OH)}_2\text{D}_2$ as the substrate concentration was raised. This indicates that in this system $1\alpha,24\text{-(OH)}_2\text{D}_2$ was the predominant natural active metabolite of $1\alpha\text{-OH-D}_2$ at higher substrate concentrations.

TABLE 1

EXPERIMENT	SUBSTRATE CONCENTRATION	PRODUCT FORMED	
1	nM	Ratio of $1\alpha,24\text{-(OH)}_2\text{D}_2$ to $1,25\text{-(OH)}_2\text{D}_2$	
	1	1:4	
	10	1:1	
	100	1.5:1	
2	μM	Rate of Production, pmol per 10^6 cells/day	
		$1\alpha,24\text{-(OH)}_2\text{D}_2$	$1,25\text{-(OH)}_2\text{D}_2$
	1	4.9	N.D.*
	10	59	7.4

*N.D. means not detectable

Example 4: Production of $1\alpha,24\text{-(OH)}_2\text{D}_2$ in osteoporotic women administered $1\alpha\text{-(OH)}_2\text{D}_2$.

An increase in the production of $1\alpha,24\text{-(OH)}_2\text{D}_2$ relative to $1,25\text{-(OH)}_2\text{D}_2$ has also been observed by the present inventors in human females who received $1\alpha\text{-OH-D}_2$ as part of an investigation of that drug for the treatment of osteoporosis. Following either a

-17-

single dose of 2 μg of $1\alpha\text{-OH-D}_2$ or daily doses of 8 $\mu\text{g/day}$ for one week, blood was collected and analyzed for the metabolites $1\alpha,24\text{-(OH)}_2\text{D}_2$ and $1,25\text{-(OH)}_2\text{D}_2$. Lipid was extracted from the blood, and the metabolites were purified by HPLC using standard methods and quantified with the radioreceptor assay produced by Incstar (Stillwater, Minnesota). One day after a single 2 μg dose, the level of $1\alpha,24\text{-(OH)}_2\text{D}_2$ was undetectable with the $1,25\text{-(OH)}_2\text{D}_2$ level being approximately 11 pg/ml. In contrast, one day following the last dose of 8 μg , the level of $1\alpha,24\text{-(OH)}_2\text{D}_2$ averaged 9 pg/ml with the $1,25\text{-(OH)}_2\text{D}_2$ level averaging 30 pg/ml.

Example 5: Affinity of $1\alpha,24\text{-(OH)}_2\text{D}_2$ for the vitamin D receptor

The affinity of $1\alpha,24\text{-(OH)}_2\text{D}_2$ for the mammalian vitamin D receptor (VDR) was assessed using a commercially available kit of bovine thymus VDR and standard $1,25\text{-(OH)}_2\text{D}_2$ solutions from Incstar (Stillwater, Minnesota). Purified $1\alpha,24\text{-(OH)}_2\text{D}_2$ was quantitated by photodiode array spectrophotometry and assayed in the radioreceptor assay. The half-maximal binding of $1\alpha,24\text{-(OH)}_2\text{D}_2$ was approximately 150 pg/ml whereas that of $1\alpha,25\text{-(OH)}_2\text{D}_2$ was 80 pg/ml. Thus, the bovine thymus VDR had a two-fold lower affinity for $1\alpha,24\text{-(OH)}_2\text{D}_2$ than for $1\alpha,25\text{-(OH)}_2\text{D}_2$, indicating that $1\alpha,24\text{-(OH)}_2\text{D}_2$ had potent biological activity.

Example 6: Affinity of $1\alpha,24\text{-(OH)}_2\text{D}_2$ for the vitamin D serum binding protein.

The affinity of $1\alpha,24\text{-(OH)}_2\text{D}_2$ for the vitamin D serum binding protein (DBP) was assessed using vitamin D deficient rat serum according to known methods in the art. The data indicated that the DBP binding of $1\alpha,24\text{-(OH)}_2\text{D}_2$ was at least 1000 times weaker than that for 25-OH-D_3 . Given the strong binding of $1\alpha,24\text{-(OH)}_2\text{D}_2$ for the VDR and weak binding for the DBP, this compound would tend to be taken up by target cells, thus possessing a potent biological activity. In addition, the weak binding by the DBP was indicative of more rapid clearance, allowing for low toxicity.

Thus, the preceding assays demonstrated that the new $1\alpha,24\text{-(OH)}_2\text{D}_2$ exhibited a distinct and unique spectrum of activities--namely, high biological potency and low toxicity

-18-

which clearly distinguished the compound from those of the prior art.

Example 7: Biological Testing

Twenty postmenopausal osteoporotic women are enrolled in an open label study. The selected patients have ages between 55 and 75 years, and exhibit L2-L3 vertebral bone mineral density between 0.7 and 1.05 g/cm², as determined by measurements with a LUNAR Bone Densitometer (Lunar Corporation, Madison, Wisconsin).

On admission to the study, all patients receive instruction on selecting a daily diet containing 400 to 600 mg of calcium. Compliance to this diet is verified at weekly intervals by 24-hour food records and by interviews with each patient.

All patients complete a one-week baseline period, a five-week treatment period, and a one-week post-treatment observation period. During the treatment period, patients orally self-administer 1 α ,24-dihydroxyvitamin D₂ at an initial dose of 0.5 μ g/day for the first week, and at successively higher doses of 1.0, 2.0, 4.0, and 8.0 μ g/day in each of the following four weeks. All doses are administered before breakfast.

Blood and urine chemistries are monitored on a weekly basis throughout the study. Key blood chemistries include fasting serum levels of calcium, phosphorus, osteocalcin, creatinine, and blood urea nitrogen. Key urine chemistries include 24-hour excretion of calcium, phosphorus, and creatinine.

Blood and urine data from this clinical study indicate that this compound does not adversely affect kidney function, as determined by creatinine clearance and blood levels of urea nitrogen; nor does it increase urinary excretion of hydroxyproline, indicating the absence of any stimulatory effect on bone resorption. The compound has no effect on any routinely monitored serum parameters, indicating the absence of adverse metabolic effects.

A positive effect of 1 α ,24-dihydroxyvitamin D₂ on calcium homeostasis is evident from modest increases in 24-hour urinary calcium levels, confirming that the compound increases intestinal calcium absorption, and from increases in serum osteocalcin levels, indicating that the compound stimulates the osteoblasts.

Example 8:

A clinical study is conducted with postmenopausal osteoporotic out-patients having ages between 55 and 75 years. The study involves up to 120 patients randomly divided into three treatment groups and continues for 24 to 36 months. Two of the treatment groups receive constant dosages of 1 α ,24-dihydroxy-vitamin D₂ (u.i.d.; two different dose levels at or above 1.0 μ g/day) and the other group receives a matching placebo. All patients maintain a normal intake of dietary calcium (500 to 800 mg/day) and refrain from using calcium supplements. Efficacy is evaluated by pre-and post-treatment comparisons of the patient groups with regard to (a) total body calcium retention, and (b) radial and spinal bone mineral density as determined by dual-photon absorptiometry (DPA) or dual-energy x-ray absorptiometry (DEXA). Safety is evaluated by comparisons of urinary hydroxyproline excretion, serum and urine calcium levels, creatinine clearance, blood urea nitrogen, and other routine determinations.

The results show that patients treated with 1 α ,24-dihydroxyvitamin D₂ exhibit significantly higher total body calcium, and radial and spinal bone densities relative to patients treated with placebo. The monitored safety parameters confirm an insignificant incidence of hypercalcemia or hypercalciuria, or any other metabolic disturbance with 1 α ,24-dihydroxyvitamin D₂ therapy.

Example 9:

A clinical study is conducted with healthy postmenopausal women having age between 55 and 60 years. The study involves up to 80 patients randomly divided into two treatment groups, and continues for 24 to 36 months. One treatment group receives a constant dosage of 1 α ,24-dihydroxy-vitamin D₂ (u.i.d.; a dose level at or above 1.0 μ g/day) and the other receives a matching placebo. The study is conducted as indicated in Example 2 above.

The results show that patients treated with 1 α ,24-dihydroxyvitamin D₂ exhibit reduced losses in total body calcium, radial or spinal bone densities relative to baseline values. In contrast, patients treated with placebo show significant losses in these parameters relative to baseline

-20-

values. The monitored safety parameters confirm the safety of long-term $1\alpha,24$ -dihydroxyvitamin D_2 administration at this dose level.

Example 10:

A twelve-month, double-blind, placebo-controlled clinical trial is conducted with thirty men and women with renal disease who are undergoing chronic hemodialysis. All patients enter an 8-week control period during which time they receive a maintenance dose of Vitamin D_3 (400 IU/day). After this control period, the patients are randomized into two treatment groups: one group receives a constant dosage of $1\alpha,24$ -dihydroxyvitamin D_2 (u.i.d.; a dosage greater than $3.0 \mu\text{g/day}$) and the other group receives a matching placebo. Both treatment groups receive a maintenance dosage of Vitamin D_3 , maintain a normal intake of dietary calcium, and refrain from using calcium supplements. Efficacy is evaluated by pre- and post-treatment comparisons of the two patient groups with regard to (a) direct measurements of intestinal calcium absorption, (b) total body calcium retention, (c) radial and spinal bone mineral density, and (d) determinations of serum calcium. Safety is evaluated by regular monitoring of serum calcium.

Analysis of the clinical data show that $1\alpha,24$ -dihydroxyvitamin D_2 significantly increases intestinal calcium absorption, as determined by direct measurements using a double-isotope technique. Patients treated with this compound show normalized serum calcium levels, stable values for total body calcium, and stable radial and spinal bone densities relative to baseline values. In contrast, patients treated with placebo show frequent hypocalcemia, significant reductions in total body calcium and radial and spinal bone density. An insignificant incidence of hypercalcemia is observed in the treated group.

Example 11: Medicament Preparations

A topical cream is prepared by dissolving 1.0 mg of $1\alpha,24$ -dihydroxyvitamin D_2 in 1 g of almond oil. To this solution is added 40 gm of mineral oil and 20 gm of self-emulsifying beeswax. The mixture is heated to liquify. After the addition of 40 ml hot water, the mixture is mixed well. The resulting

-21-

cream contains approximately 10 μg of 1 α ,24-dihydroxyvitamin D₂ per gram of cream.

Example 12:

An ointment is prepared by dissolving 1.0 mg of 1 α ,24-dihydroxyvitamin D₂ in 30 g of almond oil. To this solution is added 70 gm of white soft paraffin which had been warmed just enough to be liquified. The ointment is mixed well and allowed to cool. This ointment contains approximately 10 μg 1 α ,24-dihydroxyvitamin D₂ per gram of ointment.

Example 13:

To the ointment of Example 12 is added with thorough mixing 0.5 g of adenosine and 2.0 g of papaverine base, both dissolved in a minimum quantity of dimethyl sulfoxide. The additional ingredients are present to the extent of about 0.5 wt % (adenosine) and 2 wt % (papaverine base).

Example 14:

To the ointment of Example 12 is added with thorough mixing 10,000 U of Vitamin A dissolved in a minimum quantity of vegetable oil. The resultant ointment contains about 100 U Vitamin A per gram of the ointment.

Example 15:

A dermatological lotion is prepared by dissolving 1.0 mg of 1 α ,24-dihydroxyvitamin D₂ in 100 g of dry propylene glycol. The lotion is stored in a refrigerator in a brown bottle and contains about 10 μg of 1 α ,24-dihydroxyvitamin D₂ per gram of lotion.

Example 16:

In 1 g of almond oil is dissolved 0.2 mg of 1 α ,24-dihydroxyvitamin D₂. To the solution is added 40 g of mineral oil and 20 g of self-emulsifying beeswax, followed by 40 ml of hot water. The mixture is mixed well to produce a cosmetic cream containing about 2.0 μg of 1 α ,24-dihydroxyvitamin D₂ per gram of cream.

-22-

Example 17:

To a cosmetic cream prepared according to example 16 is added 100 mg adenosine. The cream is mixed well and contains about 0.1 wt % adenosine.

Example 18:

An ointment is prepared by dissolving 100 μ g of 1 α ,24-dihydroxyvitamin D₂ in 30 g of almond oil. To the solution so produced is added 70 g white soft paraffin which had been warmed just enough to be liquified. The ointment is mixed well and allowed to cool. The ointment so produced contains about 1.0 μ g of 1 α ,24-dihydroxy-vitamin D₂ per gram of ointment.

Example 19:

To the cosmetic ointment of Example 18 is added with thorough mixing 200 U/g Vitamin A dissolved in a minimum amount of vegetable oil.

Example 20:

A cosmetic lotion is prepared by dissolving 300 μ g of 1 α ,24-dihydroxyvitamin D₂ in 100 g of dry propylene glycol. The lotion is stored in a refrigerator in a brown bottle and contains about 3.0 μ g 1 α ,24-dihydroxyvitamin D₂ per gram of lotion.

Example 21: Dermatological Testing

Compositions containing 1 α ,24-dihydroxyvitamin D₂ are evaluated for therapeutic efficacy of the composition in the topical treatment of dermatitis (contact and ectopic). The composition evaluated is an ointment containing 10 μ g of 1 α ,24-dihydroxyvitamin D₂ per gram of ointment in a petrolatum-almond oil base. The control composition is identical except that it does not contain the active agent 1 α ,24-dihydroxyvitamin D₂. The patients are treated in an out-patient clinic. They are instructed to use the preparation two times a day.

The ointment is as far as possible applied to a single lesion, or to an area of the disease. The ointment and its container are weighed before the treatment starts and returned with any unused contents for reweighing at the end of the treatment.

-23-

The area of the lesion treated is estimated and recorded, and the lesion is photographed as required, together with suitable "control" lesions. The latter are preferably lesions of similar size and stage of development, either in the vicinity of the treated lesion or symmetrically contralateral. Relevant details of the photographic procedure are recorded so as to be reproduced when the lesions are next photographed (distance, aperture, angle, background, etc.). The ointment is applied twice daily and preferably left uncovered. The "control" lesions are left untreated, but if this is not possible, the treatment used on them is noted.

Evaluations of erythema, scaling, and thickness are conducted at weekly intervals by a physician, with the severity of the lesion rated from 0 to 3. The final evaluation is usually carried out at the end of four to six weeks of treatment. Those lesions treated with $1,24(\text{OH})_2\text{D}_3$ have lower scores than the control lesions. An insignificant incidence of hypercalcemia is also observed.

Example 22: Epidermal cell differentiation and proliferation testing

Human keratinocytes are cultured according to known modifications of the system originally described by Rheinwald and Green (Cell, 6;331 (1975)). The $1\alpha,24$ -dihydroxyvitamin D_3 , dissolved in ethanol, is added to cells to yield a variety of concentrations between 0.05 and 5 $\mu\text{g}/\text{ml}$ with the ethanol concentration not to exceed 0.5% v/v. Control cultures are supplemented with ethanol at a final concentration of 0.5% v/v.

Differentiation and proliferation of epidermal cells in culture is examined by:

1. quantitation of cornified envelopes;
2. quantitation of cell density of cells attached to disks;
3. monitoring transglutaminase activity; or
4. monitoring DNA synthesis by incorporation of ^3H -thymidine.

Cultures incubated with $1\alpha,24$ -dihydroxyvitamin D_3 have more cornified envelopes, fewer attached cells, higher

-24-

transglutaminase activity, and lower DNA synthesis than control cultures.

While the present invention has now been described and exemplified with some specificity, those skilled in the art will appreciate the various modifications, including variations, additions, and omissions, that may be made in what has been described. Accordingly, it is intended that these modifications also be encompassed by the present invention and that the scope of the present invention be limited solely by the broadest interpretation that lawfully can be accorded the appended claims.

-25-

CLAIMS

1. Use of $1\alpha,24$ dihydroxy vitamin D_2 for the preparation of a medicament for preventing or treating vitamin D deficiency-induced diseases.
2. Use of $1\alpha,24$ dihydroxy vitamin D_2 for the preparation of a medicament for the prevention or treatment of renal osteodystrophy.
3. Use of $1\alpha,24$ dihydroxy vitamin D_2 for the preparation of a topical medicament for the treatment of skin disorders.
4. Use of $1\alpha,24$ dihydroxy vitamin D_2 for the preparation of a medicament for preventing or treating depletive bone disorders.
5. A prophylactic or therapeutic pharmaceutical composition for vitamin D deficient diseases, comprising a physiologically acceptable vehicle and an effective amount of $1\alpha,24$ dihydroxy vitamin D_2 .
6. A pharmaceutical composition comprising an amount effective to prevent or treat loss of bone mass or bone mineral content in a human suffering from or predisposed to a depletive bone disorder of $1\alpha,24$ -dihydroxyvitamin D_2 in combination with a physiologically acceptable expedient.
7. A pharmaceutical composition comprising an amount effective to stabilize radial and spinal bone density in a human suffering from renal osteodystrophy of $1\alpha,24$ -dihydroxyvitamin D_2 in combination with a physiologically acceptable expedient.
8. A method for preventing or treating loss of bone mass or bone mineral content in a human experiencing or predisposed to loss of bone mass or bone mineral content, comprising administering to said human an amount of $1\alpha,24$ -dihydroxyvitamin D_2 sufficient to prevent loss of bone mass or bone mineral content without causing hypercalcemia or hypercalciuria.

-26-

9. A method for stabilizing or increasing bone mass in a human suffering from renal osteodystrophy, comprising administering to said human an amount of 1 α ,24-dihydroxyvitamin D₂ sufficient to stabilize or increase bone mass without causing hypercalcemia or hypercalciuria.

10. A composition for use in topical treatment of skin disorders, comprising a carrier suitable for topical application and 1,24-dihydroxyvitamin D₂.

11. The composition of claim 9, wherein the amount of 1,24-dihydroxyvitamin D₂ is between 0.01 μ g and 10 μ g.

12. The composition of claim 9, further comprising an agent from the group consisting of epithelialization-inducing agents, chromanols, β -agonists, anti-inflammatory agents and keratoplastic agents.

13. A method of preparing 1 α ,24 dihydroxy vitamin D₂, comprising:

- (a) acetylating ergosterol to form its 3 β -acetate;
- (b) reacting with a triazoline dione and ozonating to form the 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-chole-3 β -yl acetate;
- (c) adding 3-methylbutan-2-one to 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-chole-3 β -yl acetate to form (22E)5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl) cholesta-6,22-diene-24-one-3 β -yl acetate;
- (d) adding methylmagnesium bromide to (22E)5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl) cholesta-6,22-diene-24-one-3 β -yl acetate to form (22E)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol;
- (e) reducing the (22E)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol to form 24-hydroxy ergosterol;

-27-

- (g) irradiating 24-hydroxyergosterol to form 24-hydroxy vitamin D₂;
- (h) tosylating 24-hydroxy vitamin D₂ in the presence of dry pyridine to form 24-hydroxy vitamin D₂ 3 β -tosylate;
- (i) solvolyzing 24-hydroxy vitamin D₂ tosylate to form 24-hydroxy-3,5 cyclovitamin D₂;
- (j) allylically oxidizing the 24 hydroxy-3,5 cyclovitamin D₂ with selenium dioxide to form 1 α ,24 dihydroxy cyclovitamin D₂; and
- (k) hydrolyzing the 1 α ,24 dihydroxy 3,5 cyclovitamin D₂ with a mixture of dimethylsulfoxide and an organic acid to form an admixture of the 5,6 cis 1 α -hydroxy and 5,6 trans 1 α ,24 dihydroxy vitamin D₂ and forming a Diels-Alder adduct of the 5,6 trans 1 α -hydroxy vitamin D₂ to allow purification to yield 1 α ,24 dihydroxy vitamin D₂.

14. A method of preparing 1 α ,24 dihydroxy vitamin D₂, comprising:

- (a) tosylating 24-hydroxy vitamin D₂ to form 24-hydroxy vitamin D₂ tosylate;
- (b) methanolizing the 24-hydroxy vitamin D₂ tosylate to form 24-hydroxy 3,5 cyclovitamin D₂;
- (c) oxidizing the 24-hydroxy 3,5 cyclovitamin D₂ to form 1 α ,24-dihydroxy-3,5-cyclovitamin D₂; and
- (d) sequentially hydrolyzing and subjecting to a Diels-Alder reaction the 1 α ,24-dihydroxy-3,5 cyclovitamin D₂ to form 1 α ,24-dihydroxy vitamin D₂.

15. A method of producing 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-cholene-3 β -yl acetate, comprising reacting 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-cholene-3 β -yl acetate with 3-methylbutan-2-one in the presence of butyllithium and dry tetrahydrofuran.

16. A method of producing (22E)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol, comprising reacting 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-

-28-

1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-cholesterol-3 β -yl acetate with a Grignard reagent.

17. A method of producing 24-hydroxy ergosterol, comprising reacting (22E)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol with lithium aluminum hydride in the presence of dry tetrahydrofuran.

18. A method of producing 24-hydroxy vitamin D₂ tosylate, comprising reaction 24-hydroxy vitamin D₂ with toluenesulfonyl chloride in the presence of dry pyridine.

19. A method of producing 6-methoxy-24-hydroxy-3,5-cyclovitamin D₂, comprising solvolyzing 24-hydroxy vitamin D₂ tosylate in methanol.

20. A method of producing 6-methoxy-1 α ,24-dihydroxy-3,5-cyclovitamin D₂, comprising allylically oxidizing 6-methoxy-24-hydroxy-3,5-cyclovitamin D₂ with selenium dioxide.

21. A method for treating vitamin D deficiency-induced bone depletive diseases, comprising:

- (a) reducing ergosterol, under such conditions and in sufficient quantity to produce 24-hydroxyergosterol;
- (b) irradiating the 24-hydroxyergosterol to produce 24-hydroxy vitamin D₂;
- (c) hydroxylating the vitamin D₂ under such conditions and in sufficient quantity to produce 1 α ,24-dihydroxy vitamin D₂;
- (d) purifying the 1 α ,24-dihydroxy vitamin D₂; and
- (e) administering to a mammal suffering from a bone depletive disease an amount effective to prevent or reverse loss of bone mass or bone mineral content of 1 α ,24-dihydroxy vitamin D₂ in admixture with a pharmaceutically acceptable vehicle.

22. (22E)5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl) cholesta-6,22-diene-24-one-3 β -yl acetate.

-29-

23. (22E)-5 α ,8 α -(4-Phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol.

24. (22E)-5,7,22-Ergostatriene-3 β ,24-diol.

25. 24-Hydroxy ergosterol.

26. 24-Hydroxy vitamin D₂ tosylate.

27. 6-Methoxy-24-hydroxy-3,5-cyclovitamin D₂.

28. A method of preparing 24 hydroxy vitamin D₂, comprising:

- (a) acetylating ergosterol to form its 3 β -acetate;
- (b) reacting with a triazoline dione and ozonating to form the 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-cholesterol-3 β -yl acetate;
- (c) adding 3-methylbutan-2-one to 22-oxo-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)23,24-dinor-6-cholesterol-3 β -yl acetate to form (22E)5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)cholesta-6,22-diene-24-one-3 β -yl acetate;
- (d) adding methylmagnesium bromide to (22E)5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)cholesta-6,22-diene-24-one-3 β -yl acetate to form (22E)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol;
- (e) reducing the (22E)-5 α ,8 α -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)-6,22-ergostadiene-3 β ,24-diol to form 24-hydroxy ergosterol; and
- (g) irradiating 24-hydroxyergosterol to form 24-hydroxy vitamin D₂.

29. A feed for mammals comprising 1 α ,24 dihydroxy vitamin D₂ wherein normal consumption of the feed by the mammals provides about 0.01 to about 1.0 μ g/kg/day of 1 α ,24 dihydroxy vitamin D₂.

1/2

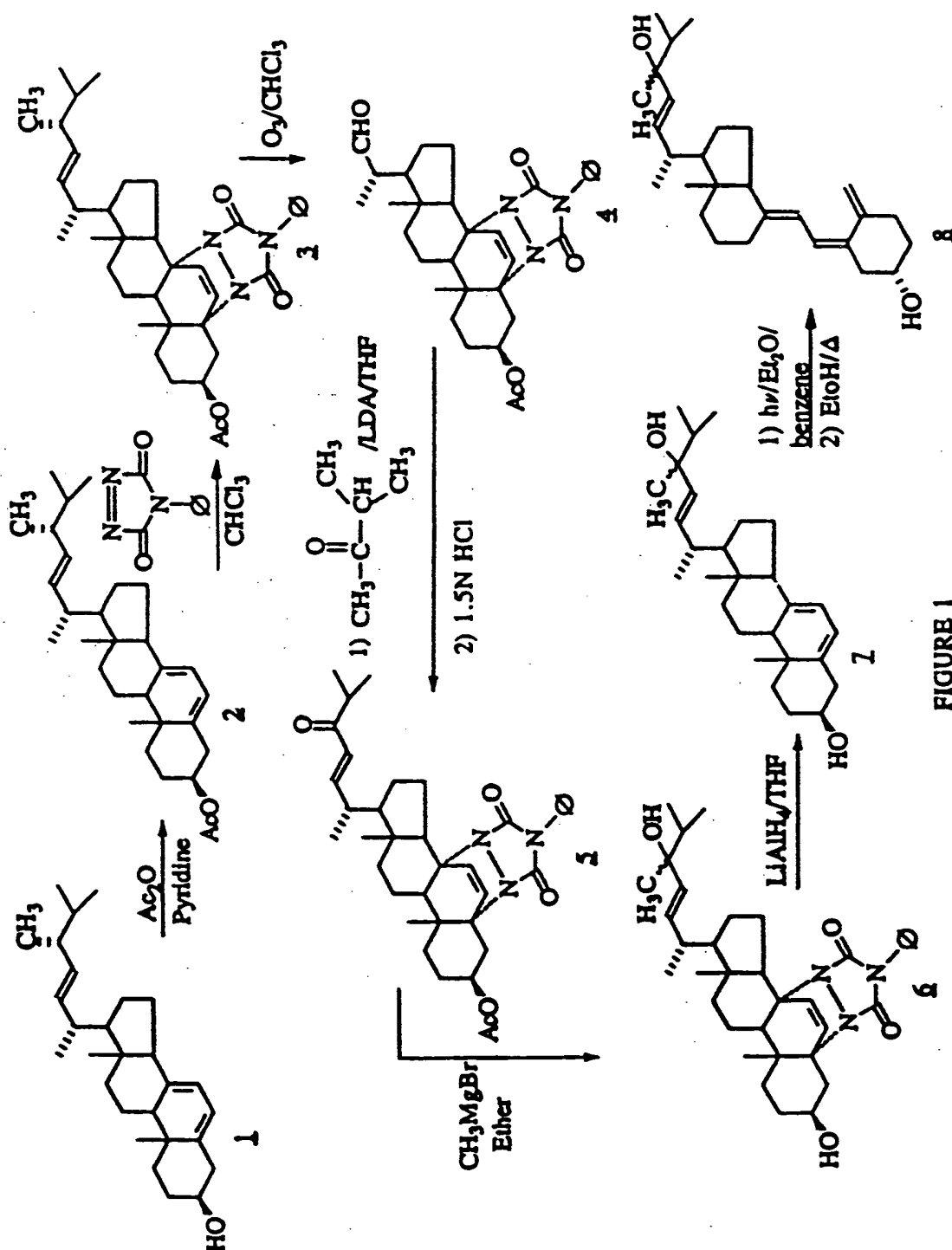


FIGURE 1

2/2

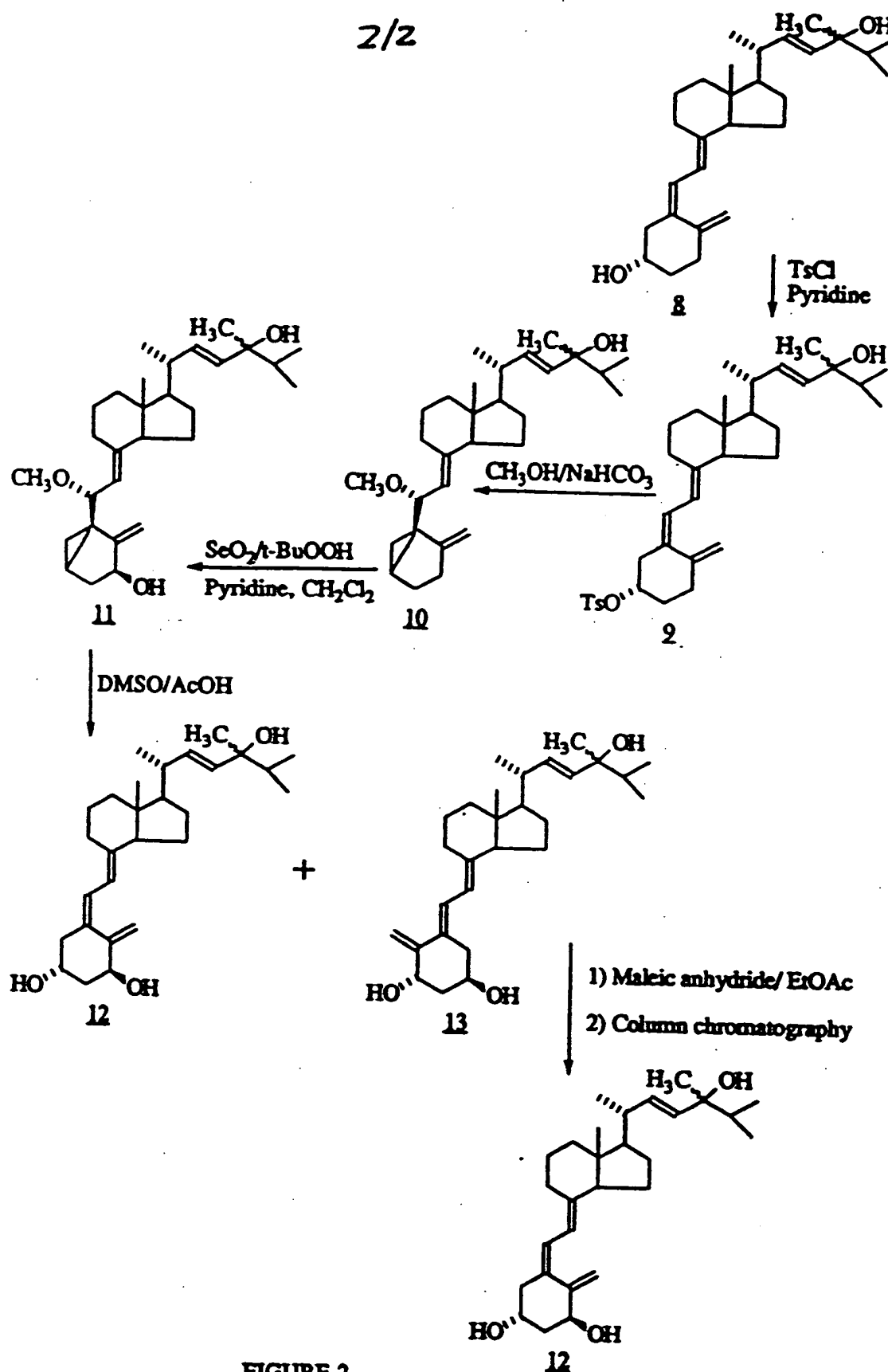
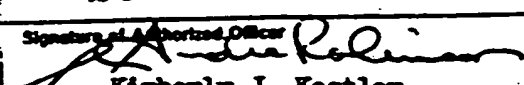


FIGURE 2

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US92/00313**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ 007J 75/ 00; A 61K 31/59 US 552/653; 514/167		
II. FIELDS SEARCHED		
Minimum Documentation Searched ?		
Classification System	Classification Symbols	
US	552/653; 514/167	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT †		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages ‡	Relevant to Claim No. §
Y	US, A, 4, 159, 326, Published 26 January 1979 Barton, et. al. See entire document	1
Y	US, A, 4, 338, 250, Published 06 January 1982 DeLuca, et. al. See entire document	1
Y,A	US, A, 4, 670, 190, Published 02 June 1987 Hesse, et. al. See entire document	Y-1 A-21
Y	US, A, 4, 195, 027, Published 25 March 1980 DeLuca, et. al. See entire document	1 1
Y	US, A, 4, 866, 048, Published 12 September 1989 Calverley, et. al. See entire document	1
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: †</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
09 April 1992		29 APR 1992
International Searching Authority		Signature of Authorized Officer
ISA/US		 Kimberly J. Kestler

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(p).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

- I. Method of treating Vitamin D deficiency - Claims 1 and 21.
- II. Method of treating renal osteodystrophy - Claims 2, 9, 11, and 12.
- III. Method of treating depletive bone disorders - Claims 4 & 8
- IV. Method of treating skin disorders - Claim 3

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1 and 21

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4, 448, 721, Published 15 May 1984 DeLuca, et. al. See entire document.	1 and 21
A	US, A, 4, 758, 383, Published 19 July 1998 Tachibana. See entire document.	1 and 21
A	USA 4, 310, 511, Published 12 January 1982 Holick. See entire document.	1 and 21

VI. Observations Where Unity Of Invention Is Lacking²
(Continued from fourth sheet)

- V. Composition for treating Vitamin D deficient diseases - Claim 5
- VI. Composition for treating loss of bone mass or bone mineral content - Claim 6
- VII. Composition for treating osteodystrophy - Claim 7
- VIII. Composition for topical treatment of skin disorders - Claim 10
- IX. Method of making 1 , 24-dihydroxy Vitamin D - Claim 13
- X. Second method of making 1 , 24-dihydroxy Vitamin D - Claim 14
- XI. Method of making 22-oxo-5 , 8 -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)
23,24-dinor-6-cholesterol-3B-yl acetate - Claim 15
- XII. Method of making (22E)-5 , 8 -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)
-6,22-ergostadiene-3B-24-diol- Claim 6
- XIII. Method of making 24-hydroxy ergosterol - Claim 17
- XIV. Method of making 24-hydroxy Vitamin D tosylate - Claim 18
- XV. Method of making 6-methoxy-24-hydroxy-3,5-cyclovitamin D - Claim 19
- XVI. Method of making 6-methoxy-1 ,24-dihydroxy-3,5-cyclovitamin D - Claim 20
- XVII. Compound:(22E) 5 ,8 -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2,-diyl)
cholesterol-6,22-diene-24-one-3B-yl acetate - Claim 22
- XVIII. Compound:(22E)-5 ,8 -(4-phenyl-3,5-dioxo-1,2,4-triazolidine-1,2-diyl)
-6,22-ergostadiene-3B,24-diol - Claim 23
- XIX. Compound:(22E)-5,7,22-Ergostatriene-3B,24-diol - Claim 24
- XX. Compound:24-hydroxy ergosterol - Claim 25
- XXI. Compound:24-hydroxy Vitamin D tosylate - Claim 26
- XXII. Compound:6-methoxy-24-hydroxy-3,5-cyclovitamin D - Claim 27
- XXIII. Method of making 24 hydroxy Vitamin D - Claim 28
- XXIV. Feed for mammals - Claim 29

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)